Adenosine A_3 agonists reverse neuropathic pain via T cell–mediated production of IL-10

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The A_3 adenosine receptor (A_3AR) has emerged as a therapeutic target with A_3AR agonists to tackle the global challenge of neuropathic pain, and investigation into its mode of action is essential for ongoing clinical development. Immune cell A_3ARs, and their activation during pathology, modulate cytokine release. Thus, the use of immune cells as a cellular substrate for the pharmacological action of A_3AR agonists is enticing, but unknown. The present study discovered that *Rag*-KO mice lacking T and B cells, as compared with WT mice, are insensitive to the anti-allodynic effects of A_3AR agonists. Similar findings were observed in interleukin-10 and interleukin-10 receptor knockout mice. Adoptive transfer of $CD4^+$ T cells from WT mice infiltrated the dorsal root ganglion (DRG) and restored A_3AR agonist-mediated anti-allodynia in *Rag*-KO mice. $CD4^+$ T cells from *Adora3*-KO or *Il10*-KO mice did not. Transfer of $CD4^+$ T cells from WT mice, but not *Il10*-KO mice, into *Il10*-KO mice or *Adora3*-KO mice fully reinstated the anti-allodynic effects of A_3AR activation. Notably, A_3AR agonism reduced DRG neuron excitability when cocultured with $CD4^+$ T cells in an IL-10–dependent manner. A_3AR action on $CD4^+$ T cells infiltrated in the DRG decreased phosphorylation of GluN2B-containing N-methyl-D-aspartate receptors at Tyr1472, a modification associated with regulating neuronal hypersensitivity. Our findings establish that activation of A_3AR on $CD4^+$ T cells to release IL-10 is required and sufficient evidence for the use of A_3AR agonists as therapeutics.

Introduction

Chronic neuropathic pain constitutes a large unmet medical need affecting 15–30 million people in the United States (1), and the annual economic burden cannot be underscored (2). Neuropathic pain arises when peripheral nerves are injured by trauma, disease, or toxins. Neuropathic pains are chronic, severe, debilitating, and exceedingly difficult to treat with currently available analgesics (3). Novel nonnarcotic analgesics are needed. Recently, the Gi-coupled A_3 adenosine receptor (A_3AR) was identified as a novel target for therapeutic intervention with selective A_3AR agonists (4–6). Continued investigation into their mode of action is essential, as these are in clinical development. Human and rodent immune cells, and in particular T cells (including $CD4^+$ and $CD8^+$), express high A_3AR levels (7), but whether these receptors play a role in the beneficial agonist effects in neuropathic pain is unknown. Interestingly, A_3AR activation on circulating immune cells harvested from animal models of autoimmune disorders blocks the formation of neuroexcitatory/inflammatory cytokines such as TNF and interleukin 1β and enhances interleukin-10 (IL-10) release (8). Similar findings were obtained with immune cells harvested from patients with autoimmune disorders validating the target in humans (9, 10). IL-10 is a potent anti-inflammatory and neuroprotective cytokine with documented positive effects in mitigating neuropathic pain (11, 12). These data, in parallel fields of studies, point to a potential link between immune cells and IL-10 in A_3AR agonist action. Using behavioral, genetic, pharmacological, and electrophysiological approaches, the present study explores the contribution of T cells to the pharmacological actions of A_3AR agonists in traumatic nerve injury–induced neuropathic pain.

Results and Discussion

Mouse sciatic nerve chronic constriction injury (CCI) leads to neuropathic pain (mechano-allodynia) that is maximal by day 7 (D7) and maintained for several weeks after injury (13). Intraperitoneal injection of highly selective A_3AR agonist MRS5980 at time of peak neuropathic pain reverses mechano-allodynia in both female and male mice (Figure 1A and Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI139299DS1), with effects lost in mice

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Conflict of interest: DS is founder of BioIntervene Inc., a company that is developing A_3AR agonists for clinical use.

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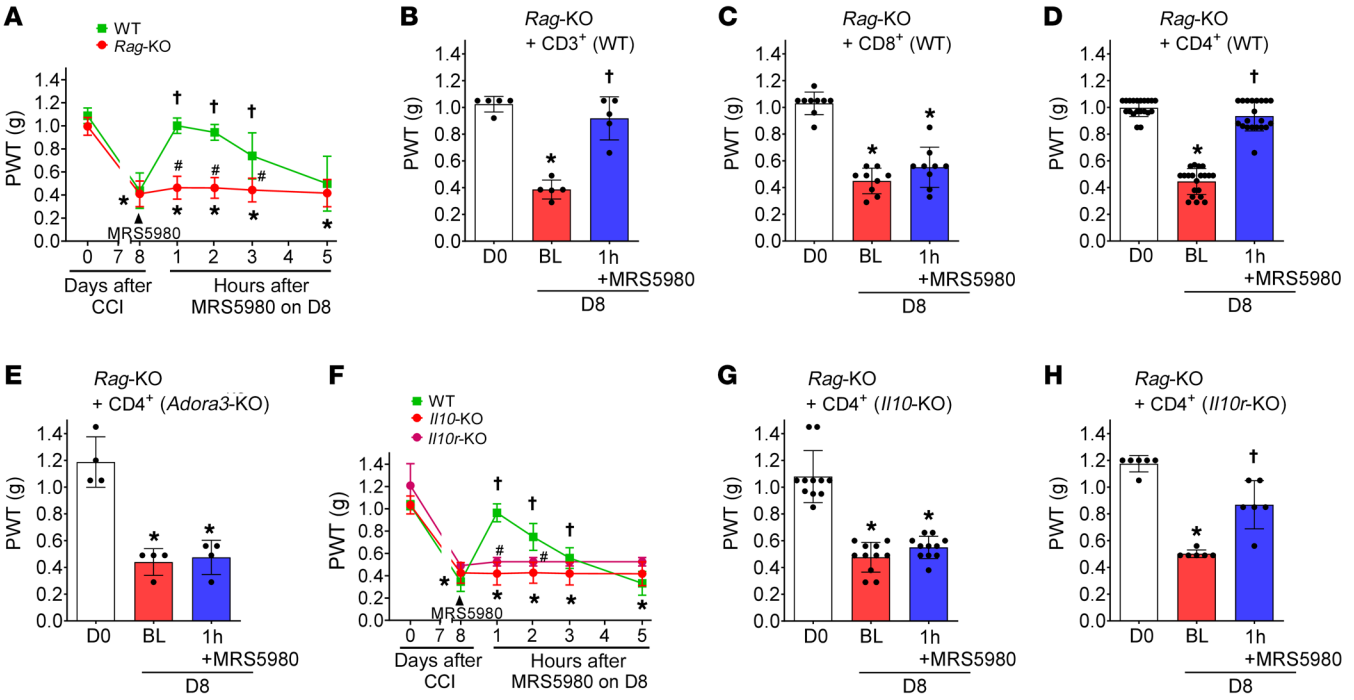

Figure 1. Anti-allodynic effects of A_3AR agonists require CD4⁺ T cells. (**A**) Injection of MRS5980 (1 mg/kg, i.p.) given at time of peak neuropathic pain reversed allodynia in male and female WT mice (n = 7 males and n = 5 females) but not *Rag*-KO mice (n = 6 males and n = 5 females). Adoptive transfer of CD3⁺ (**B**, n = 5) or CD4⁺ (**D**, n = 15 males and n = 5 females) T cells but not CD8⁺ T cells (**C**, n = 9) from WT mice into *Rag*-KO mice restored the anti-allodynic effects of MRS5980. Adoptive transfer of CD4⁺ T cells from *Adora3*-KO mice (**E**, n = 4) failed to restore the anti-allodynic effect of MRS5980. Injection of MRS5980 (1 mg/kg, i.p.), ipsilateral to nerve injury during peak mechano-allodynia reversed allodynia in WT mice (n = 5) but not in *Il10*-KO (n = 5) or *Il10r*-KO (n = 6) mice (**F**). The anti-allodynic effect of MRS5980 lost in both male and female *Rag*-KO mice after adoptive transfer of CD4⁺ T cells from *Il10*-KO mice (**G**, n = 6 males and n = 5 females) was restored after adoptive transfer of CD4⁺ T cells from *Il10r*-KO mice (**H**, n = 9). Data are mean ± SD (**A**–**H**). $^*P < 0.05$ vs. D0; $^\dagger P < 0.05$ vs D8/BL by 2-way repeated measures ANOVA (**A** and **F**) or 1-way ANOVA with Dunnett's pair-wise comparisons (**B**–**E**, **G**, and **H**). $^\#P < 0.05$ vs. WT by 2-way repeated measures ANOVA with Sidak (**A**) or Tukey's (**F**) pair-wise comparisons.

deficient in T and B cells (*Rag*-KO mice) (Figure 1A and Supplemental Figure 4). No significant difference in mechano-allodynia between WT and *Rag*-KO mice after nerve injury was observed, confirming previous studies (14). A_3AR agonist doses were chosen from our previous studies to cause a near-to-maximal reversal of mechano-allodynia in this model (15). Adoptive transfer (D7 after CCI) of CD3⁺ T cells from WT mice restored the A_3AR agonist effects in *Rag*-KO mice (Figure 1B and Supplemental Figure 1). CD8⁺ T cell adoptive transfer from WT mice did not restore A_3AR agonist anti-allodynic effects in *Rag*-KO mice. In contrast, adoptive transfer of CD4⁺ T cells fully reinstated anti-allodynic effects in both male and female *Rag*-KO mice (Figure 1, C and D and Supplemental Figure 1). CD4⁺ T cell adoptive transfer from A_3AR knockout (*Adora3*-KO) mice failed to restore the anti-allodynic effects of A_3AR agonists in *Rag*-KO mice, indicating that A_3AR activation on CD4⁺ T cells is required for A_3AR agonist anti-allodynic activity (Figure 1E and Supplemental Figure 1). The anti-allodynic responses to morphine were unaltered in *Rag*-KO mice compared with WT mice (Supplemental Figure 2), confirming that a lack of anti-allodynic responses is not a general, nonspecific response.

These results suggest that, following A_3AR activation, CD4⁺ T cells release mediators that rapidly reverse allodynia. Therefore, we focused on IL-10, which can be released by T cells (16) and is able to reverse neuropathic pain states (17, 18). Moreover, neurons as well as both CD4⁺ T cells and CD8⁺ T cells express A_3AR, IL-10, and IL-10R (19, 20). The anti-allodynic effects exerted by A_3AR agonists were lost in *Il10*-KO and in IL-10 receptor (*Il10r*-KO) mice (Figure 1F and Supplemental Figure 1). Thus, an intact IL-10/IL-10R system is required for A_3AR agonist effect (5). In order to test whether CD4⁺ T cells are a source of IL-10, we examined A_3AR agonist responses in *Rag*-KO mice that were adoptively transferred with CD4⁺ T cells from *Il10-KO* mice. In both male and female *Rag-KO* mice repopulated with CD4⁺ T cells from *Il10-KO* mice, A_3AR agonists failed to reverse mechano-allodynia (Figure 1G and Supplemental Figure 1), establishing CD4⁺ T cells as the predominant IL-10 source. In contrast, the A_3AR agonist anti-allodynic effects were uncompromised in *Rag-KO* mice reconstituted with CD4⁺ T cells from *Il10r*-KO mice (Figure 1H and Supplemental Figure 1). Collectively, the data suggest that CD4⁺ T cell–derived IL-10, but not the presence of IL-10 receptor on the CD4⁺ cells, is necessary for the effects of A_3AR agonists.

In *Il10*-KO mice, adoptive transfer of CD4⁺ T cells from WT but not *Il10*-KO mice restored the anti-allodynic effects of A_3AR agonists (Figure 2, A–C and Supplemental Figure 1). This information supports similar findings in *Rag*-KO mice and the premise that CD4⁺ T cell–derived IL-10 is necessary for A_3AR agonist effects.

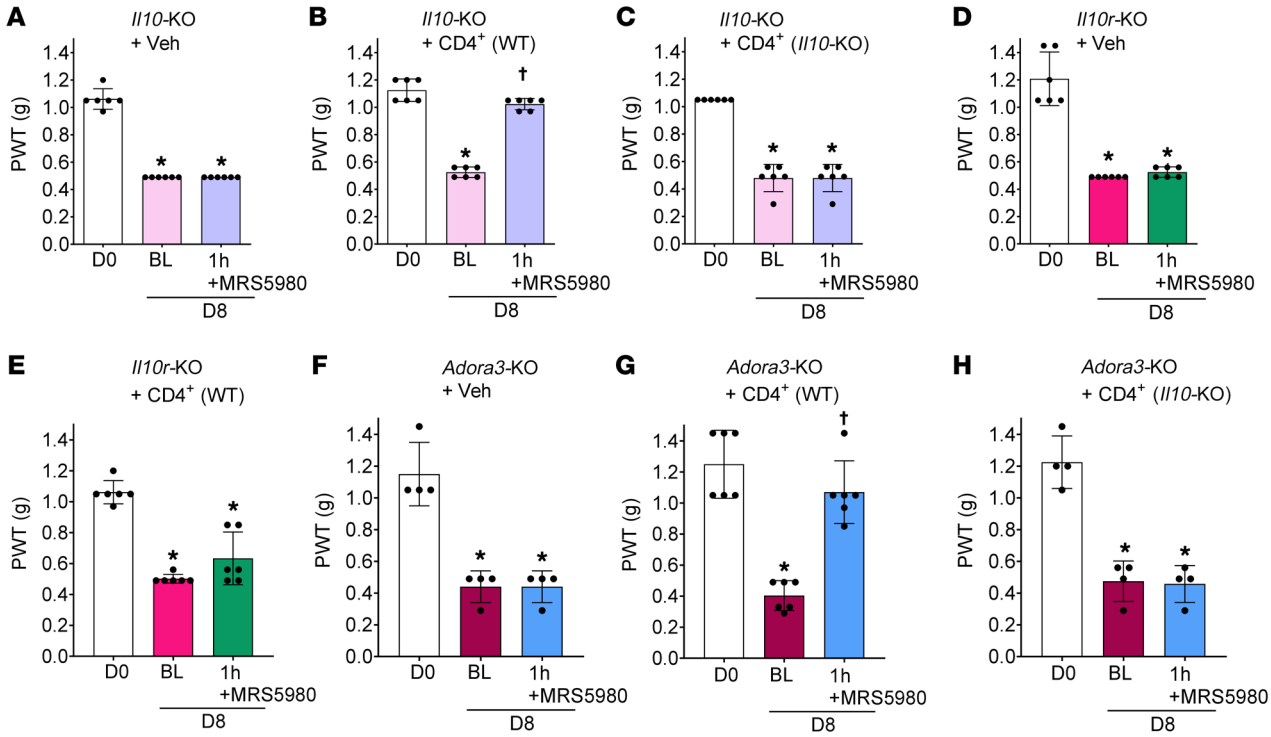

Figure 2. Activation of A_3AR expressed on CD4⁺ T cells is required in the anti-allodynic effects of A_3AR agonist; role of IL-10. The anti-allodynic effects of MRS5980 were lost in *Il10*-KO (**A**, *n* = 6) and *Il10r*-KO (**D**, *n* = 6) mice. CD4⁺ T cell adoptive transfer from WT mice (**B**, *n* = 6) but not from *Il10*-KO mice (**C**, *n* = 6) restored the anti-allodynic effects of MRS5980 in *Il10*-KO mice. Conversely, adoptive transfer of CD4⁺ T cells from WT mice (**E**, *n* = 6) did not restore the anti-allodynic effects of MRS5980 in *Il10r*-KO mice. The anti-allodynic effects of MRS5980 were lost in *Adora3*-KO mice (**F**, *n* = 4). Adoptive transfer of CD4⁺ T cells from WT mice (**G**, *n* = 6) but not from *Il10*-KO mice (**H**, *n* = 4) restored the anti-allodynic effects of MRS5980 in *Adora3*-KO mice. Data are mean ± SD. **P* < 0.05 vs. D0; †*P* < 0.05 vs. D8/BL by 1-way ANOVA with Dunnett's pair-wise comparisons.

A_3AR effects lost in *Il10r*-KO mice are not restored by adoptive transfer of WT CD4⁺ T cells (Figure 2, D and E and Supplemental Figure 1), reinforcing the notion that CD4⁺ T cell–derived IL-10 is essential in A_3AR agonists' mode of action.

To determine whether A_3AR activation on CD4⁺ T cells is required and sufficient for the IL-10 response, behavioral outcomes in *Adora3*-KO mice were investigated. A_3AR agonists did not reverse mechano-allodynia in *Adora3*-KO mice (Figure 2F and Supplemental Figure 1). However, adoptive transfer of CD4⁺ T cells from WT donors but not from IL-10–KO mice into *Adora3*-KO mice completely restored the agonists' anti-allodynic effects (Figure 2, G and H and Supplemental Figure 1). These results establish that A_3AR activation on CD4⁺ T cells drives the IL-10 response. As previously described (5, 21, 22), we observed no reduction of mechano-allodynia in *Il10*-KO and *Il10r*-KO mice compared with WT mice. Moreover, the anti-allodynic responses to morphine were not altered in *Il10*-KO (23) and *Adora3*-KO (24) mice compared with WT mice. No changes in contralateral paws were observed in any study (Supplemental Figures 3 and 4).

The hypersensitivity of primary sensory neurons that develops in the DRG is critically important in neuropathic pain development (25), and increased phosphorylation of GluN2B-containing N-methyl-D-aspartate receptors (NMDARs) at Tyr1472 [GluN2B(Tyr1472)] contributes to this increase (26, 27). Our data suggest that CD4⁺ T cell infiltration in the DRG attenuates neuronal excitability following A_3AR activation. A- and C-type DRG neurons express the IL-10 receptor (alpha subunit, IL-10RA) (28). IL-10 can block phosphorylation of NMDARs by attenuating NMDA-induced intracellular calcium concentration increases (29), inhibiting protein kinases and phosphatases known to regulate NMDAR channel activity (30), inhibiting DRG neuronal firing (28, 31), and reducing neuronal firing indirectly by have effects on non-neuronal cells (11). Consistently, application of IL-10 to DRG neurons isolated from naive mice prevented action potential (AP) initiation (Figure 3, A–C, Supplemental Figure 5). Of note, DRG neurons exposed to IL-10 were still able to respond to the transient receptor potential vanilloid 1 (TPV1) agonist capsaicin (Supplemental Figure 6).

Our study next examined whether A_3AR agonism leads to inhibition of DRG neuronal excitability via IL-10 release from CD4⁺ T cells. Immunofluorescence analysis of DRGs harvested from *Rag*-KO mice following adoptive transfer of CD4⁺ T cells from WT mice expressing enhanced green fluorescence protein (GFP) showed increased CD4⁺ T cell numbers in DRG ipsilateral to nerve injury compared with those that were contralateral (Figure 3, D and E). The A_{2A}AR receptor subtype, not the A_3AR, seems to have the predominant role in lymphocyte migration (32, 33). Furthermore, in C57BL/6 mice, it has been reported that the absence of IL-10 receptor on the CD4⁺ T cell surface does not impair trafficking in inflammatory conditions,

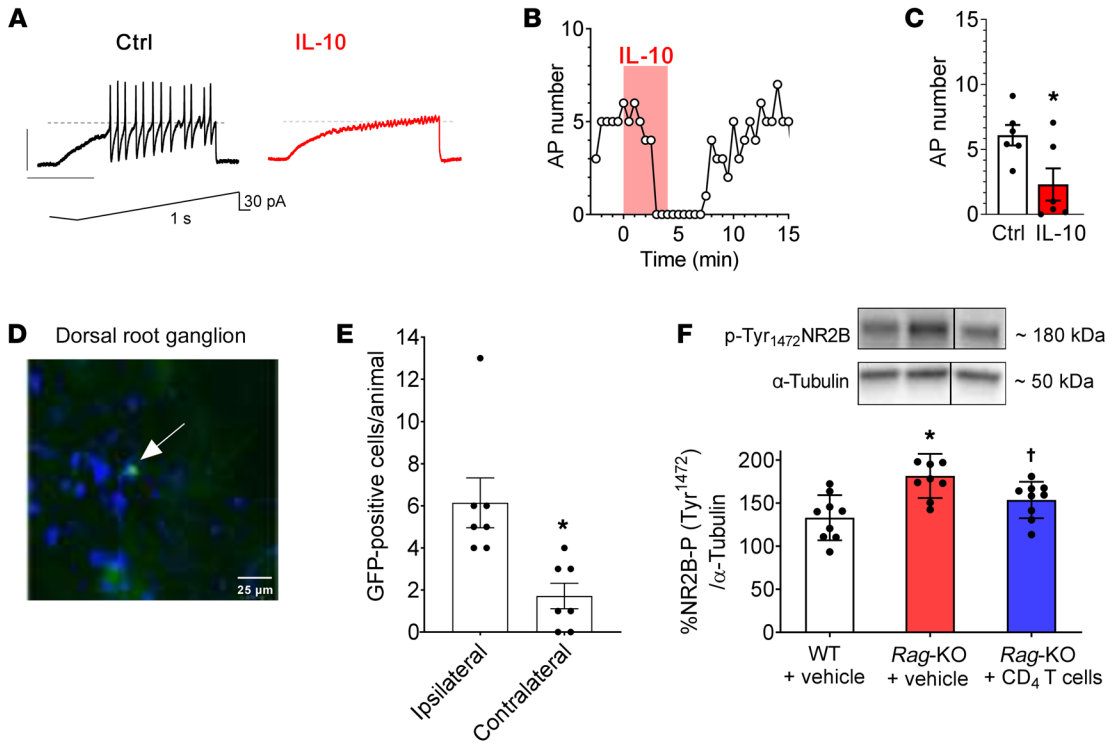

Figure 3. Functional effects of IL-10 on cell firing in DRG neurons, and CD4⁺ T cell infiltration in mouse DRG neurons. (**A**) Original current-clamp traces recorded by whole-cell patch-clamp technique in a typical naive mouse DRG neuron where IL-10 (0.5 μg/mL) reversibly inhibits AP firing evoked by a depolarizing ramp current injection (1 second; 30 pA; lower inset) once every 30 seconds. Dotted lines indicate the 0 mV level. The number of APs elicited by the current ramp was plotted as a function of time in the same cell (**B**) or was expressed as pooled data (mean ± SEM) in the bar graph (**C**, $n = 6$). *$P = 0.0018$, paired Student's t test; scale bars: 300 ms; 50 mV (**C**). CD4⁺ T cells (arrow) (magnification ×40) are present in the ipsilateral DRG of the *Rag-KO* mice reconstituted with CD4⁺ T cells from WT GFP mice (green, GFP; blue, DAPI) (**D**, **E**; $n = 7$). MRS5980 reduced Tyr1472 phosphorylation of GluN2B in the DRG of *Rag*-KO mice after adoptive transfer of CD4⁺ T cells from WT mice (**F**, $n = 9$). Density of each p-Tyr$_{1472}$GluN2B band was calculated relative to α-tubulin. Data are mean ± SEM (**E**) or mean ± SD (**F**). *$P < 0.05$. WT+veh or ipsilateral; †$P < 0.05$ vs. *Rag*-KO+veh by 2-tailed Student's t test (**E**) or 1-way ANOVA (**F**) with Dunnett's pair-wise comparisons.

suggesting the IL-10/IL-10R system is nonessential for T cell migration (34). So, although A_3AR activation of IL-10 inhibition may affect T cell migration, we consider this to be unlikely. Intraperitoneal injection of MRS5980 caused a significant decrease in GluN2B(Tyr1472) phosphorylation in DRG ipsilateral to nerve injury in *Rag*-KO mice after CD4⁺ T cell adoptive transfer from WT mice compared with *Rag*-KO mice with no adoptive transfer (Figure 3F).

To explore potential cross-talk between CD4⁺ T cells and neurons in the DRG, we performed an in vitro study, coculturing primary mouse DRG neurons with primary mouse CD4⁺ T cells—both cell types isolated from naive animals. A_3AR agonist MRS5980 significantly decreased the number of APs evoked by a 30 pA ramp current in DRG neurons when cocultured with CD4⁺ T cells (Figure 4, A–C). Concurrently, a marked increase in current threshold (Supplemental Table 1) was detected. These effects were prevented by an anti-IL-10 antibody (Figure 4, D–F) but not by a control IgG isotype (Supplemental Figure 7) and were not observed when DRG neurons were cocultured with CD8⁺ T cells (Figure 4, G–I). MRS5980 did not alter cell excitability when CD4⁺ T cells were absent in the DRG culture (Figure 4, J–L). This result is at variance with findings that we recently published, in which A_3AR activation reduced neuronal firing in rat DRG neurons (35). This difference is possibly due to the reported lack of A_3AR expression in mouse DRG neurons (36, 37). When mouse DRG neurons were cultured in the absence of CD4⁺ T cells (Figure 4L), cocultured with CD4⁺ T cells (Figure 4C), cocultured with CD4⁺ T cells with anti-IL-10 antibody present (Figure 4F), or cocultured with CD8⁺ T cells (Figure 4I), the number of APs elicited by the current ramp in control conditions (before MRS5980 application) was similar among the groups. Results were replicated in DRG and CD4⁺ T cells isolated from CCI animals on D7 (Supplemental Figure 8 and Supplemental Table 1). Of note, DRG neurons isolated from CCI mice presented a markedly smaller current threshold to first AP (Supplemental Table 1), so ramp current injection was lowered to 15 pA to avoid signal saturation (Supplemental Figure 8).

Collectively, these results suggested a model whereby A_3AR agonists reverse established hypersensitivity by activating A_3AR expressed on CD4⁺ T cells to release IL-10, reducing neuronal DRG excitability (Graphical Abstract).

Methods

Detailed experimental methods are included with the Supplemental Material.

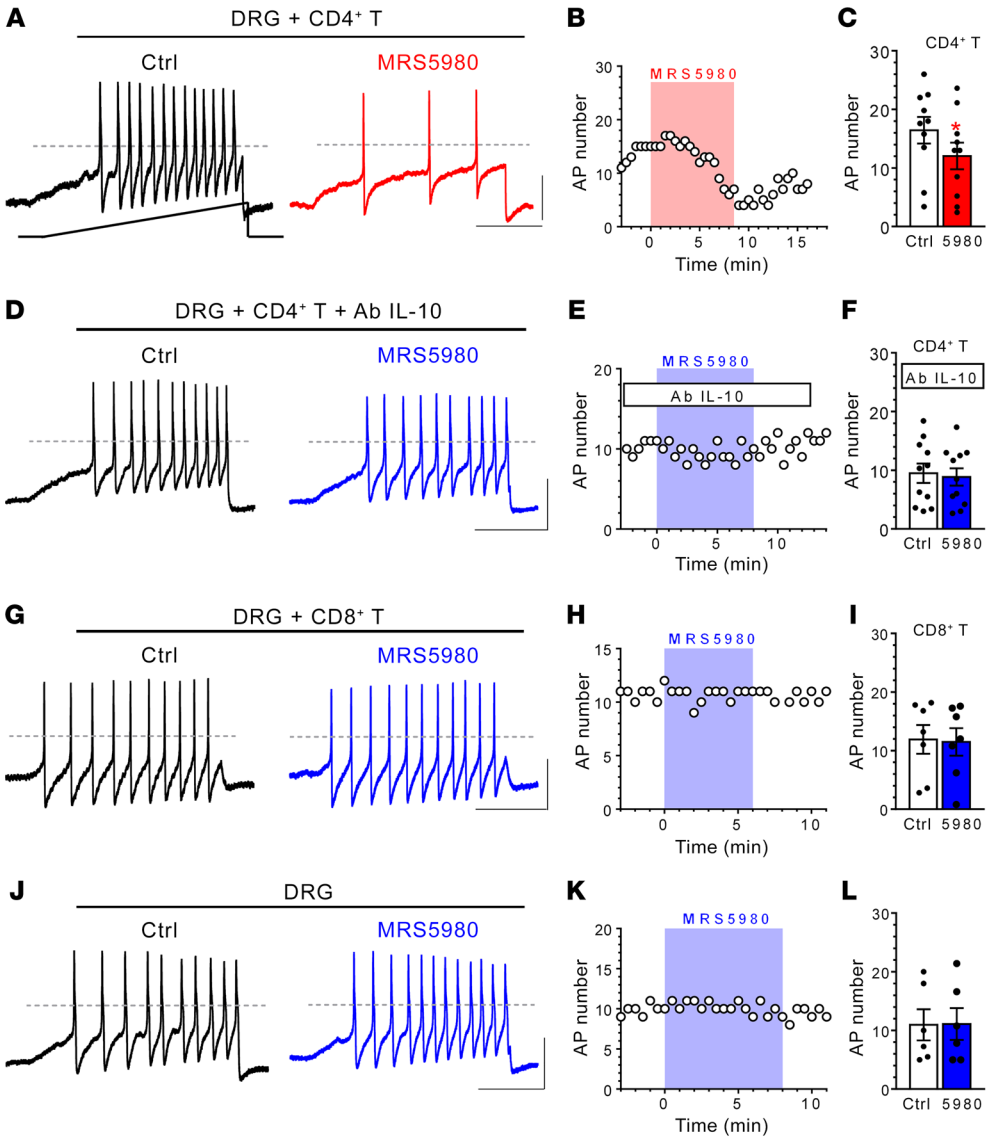

Figure 4. IL-10 released by CD4⁺ T cells is required for A_3AR agonist–mediated inhibition of AP firing in cocultured mouse DRG neurons isolated from naive mice. Original current-clamp traces recorded by whole-cell patch-clamp technique in typical mouse DRG neurons. AP firing was evoked by a depolarizing ramp current injection (1 second; 30 pA; lower inset) once every 30 seconds. The A_3AR agonist MRS5980 (300 nM) was applied in (**A**) DRG-CD4⁺ T cell cocultures, (**D**) DRG-CD4⁺ T cell cocultures in the presence of anti-IL-10 antibody (Ab IL-10; 0.5 μg/ml), (**G**) DRG-CD8⁺ T cell cocultures, and (**J**) DRG cultures. The number of APs elicited by the current ramp was plotted as a function of time in 4 different representative cells (**B**, **E**, **H**, and **K**) or was expressed as pooled data (mean ± SEM) in the bar graphs (**L**, $n = 6$; **C**, $n = 10$; **F**, $n = 11$; **I**, $n = 7$). Dotted gray lines indicate the 0 mV level. *$P = 0.0120$, paired Student's t test. The number of APs elicited before MRS5980 application (with bars, ctrl) was not different in DRG neurons cultured alone (**L**), DRG neurons cocultured with CD4⁺ T cells (**C**), DRG neurons cocultured with CD4⁺ T cells in the presence of anti-IL-10 antibody (**F**), or DRG neurons cocultured with CD8⁺ T cells (**I**). One-way ANOVA with Bonferroni comparison: **L** vs **C**: $P = 0.3981$; **C** vs. **F**: $P = 0.1034$; **L** vs. **F**: $P > 0.9999$; **L** vs. **I**: $P > 0.9999$. Scale bars: 300 ms; 50 mV.

Study approval. All animal procedures followed NIH guidelines and European Economic Community (86/609/CEE) recommendations. Experiments were approved by the Saint Louis University IACUC and by the University of Florence Animal Ethical and Care Committee.

Author contributions

DS conceived and designed the studies. EC designed the electrophysiology studies. MD, FL, SS, LDCM, CG, GK, CX, CW, LAG, FC, and EC performed the experiment and analysis. DKT, MLR, and KAJ provided key reagents. AO and DH provided technical input. DS, MD, SS, and FL prepared the manuscript with input from all authors. The order of the first co–authors was determined by degree of involvement with the project; MD and SS were involved during the project's pilot phase; FL joined during the project's maturation.

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